FEBS Letters 580 (2006) 1801–1806

Normal specification of the extraembryonic lineage after somatic nuclear transfer

Satoshi Kishigami*, Takafusa Hikichi, Nguyen Van Thuan, Hiroshi Ohta, Sayaka Wakayama, Hong-Thuy Bui, Eiji Mizutani, Teruhiko Wakayama

Laboratory for Genomic Reprogramming, Center for Developmental Biology RIKEN Kobe, 2-2-3 Minatojima-minamimachi, Kobe 650-0047, Japan

Received 13 December 2005; revised 30 January 2006; accepted 15 February 2006

Available online 24 February 2006

Edited by Michael R. Bubb

Abstract To examine the establishment and maintenance of trophectoderm (TE) lineage in somatic cloned blastocysts, the expression of Cdx2, a key molecule for specification of TE fate, was immunohistochemically examined simultaneously with Oct4 expression. Cloned mouse embryos were made by nuclear transfer using cumulus cells, tail-tip fibroblasts, and embryonic stem cells. After 96 h of culture, the rates of Oct4-expressing blastocysts were as low as 50% and 60% for cumulus and fibroblast clones, respectively. However, regardless of Oct4 expression, the majority of those cloned blastocysts (>90%) normally expressed Cdx2. Thus, even though somatic cloned embryos have reduced potential to produce the inner cell mass lineage, the TE lineage can be established and maintained.

Keywords: Mouse; Nuclear transfer; Preimplantation; Cdx2; Oct4

1. Introduction

The success of somatic cell cloning in the mouse gives promise to applications such as species preservation, livestock propagation, and cell therapy for medical treatment by nuclear transfer embryonic stem (NT-ES) cells [1–4]. However, cloning by somatic cell nuclear transfer (SCNT) is still inefficient. During development, a significant number of cloned embryos die during the pre- and peri-implantation period [5]. This early stage lethality may represent a potential problem when establishing either or both embryonic and extraembryonic lineages within the developing embryo.

Blastocysts are composed of the inner cell mass (ICM) that will form the embryo proper, and the trophectoderm (TE) that forms the trophoblast lineage for the future placenta. The molecular mechanism underlying the initial establishment of the TE and ICM lineages has been elusive. *Oct4* (*Pou5f1*-Mouse Genome Informatics) encodes a POU-domain transcription factor which is required for maintenance of the ICM as well as pluripotency of ES cells [6,7]. By the morula stage, *Oct4* is expressed in all blastomeres. But, after initiation of blastocyst formation, *Oct4* becomes restricted to the ICM and downregulated in the TE [8,9]. Since *Oct4*-deficient embryos fail to form the pluripotent ICM, it can be surmised that embryos require Oct4 for establishment of the normal ICM as well as its maintenance [6]. Further, conditional repression of *Oct4* in ES cells leads to induction of TE markers including *Cdx2* [7,10]. Cdx2, a caudal-type homeodomain transcription factor, is the earliest transcription factor to be involved in specification of TE fate expressing at the morula stage and then restricted to the TE at the blastocyst stage [9,11]. Homozygous mutant *Cdx2* embryos fail to maintain the blastocoel, have reduced expression of TE markers including *Eomes* and then, die around the time of implantation [11,12]. Further, *Cdx2* mutant blastocysts exhibit the expanded expression of *Oct4* and *Nanog* in the presumptive TE. Together with these findings, the recent accumulating evidence strongly suggest that Oct4 and Cdx2 are mutually inhibitory either directly or indirectly [13,14] and indicate that these two genes are not only the lineage markers but also essential for the establishment and maintenance of the ICM and TE lineages at the blastocyst stage.

In somatic cloned blastocysts, *Oct4* is often downregulated or abnormally expressed in presumptive TE cells, suggesting the loss of or reduced pluripotency in the ICM lineage in cloned embryos [15–17]. However, it has not been well examined how normally the extraembryonic lineage is established and maintained in somatic cloned embryos in the presence of the abnormal ICM lineage. Therefore, using anti-Cdx2 and anti-Oct4 antibodies we address how normally somatic cloned blastocysts establish and maintain the extraembryonic lineage with emphasis on the spatial patterning of Cdx2 expression.

2. Materials and methods

2.1. Animals

B6D2F1 mice (C57BL/6 × DBA/2) were used to prepare oocyte and somatic-cell (cumulus or fibroblast cells) donors as well as spermatozoa.

2.2. Cloned embryos by using adult somatic cells and microinsemination (ICSI)

Nuclear transfer was performed as described [18]. Enucleated B6D2F1 oocytes were injected individually with an adult tail-tip, cumulus cell, or ES cell (E14) nuclei [18–20]. After nuclear transfer, the reconstructed oocytes were activated by Sr2+ and cultured for 4 days in KSOM medium. Collection of spermatozoa as well as ICSI was performed according to previously described methods [21].

*Corresponding author. Fax: +81 78 306 3095.
E-mail address: kishigami@cdb.riken.jp (S. Kishigami).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.036

2.3. Indirect immunofluorescence

All donor cells were fixed after plating except cumulus cells which were fixed by direct suspension in 4% paraformaldehyde. Fertilized embryos used as controls were made by ICSI and cultured in KSOM until 3.5 dpc and then fixed following attachment and outgrowth of 3 days after plating in DMEM containing 20% FCS.

For analysis of expanded blastocysts, following oocyte activation after nuclear transfer or ICSI, embryos were cultured for 96 h. Either expanding or expanded blastocysts with more than 20 cells were fixed overnight at 4 °C in phosphate-buffered saline containing 4% paraformaldehyde. The following process is as described previously [22]. Primary antibodies against Oct4 (H-134, Santa Cruz Biotechnology, CA, USA) and Cdx2 (CDX2-88, BioGenex, CA, USA), were used.

2.4. RT-PCR analysis

RT-PCR reactions to detect *Oct4*, *Cdx2*, and *Hprt* were performed basically following the previous report [23] with small changes in cDNA preparation and the use of Taq DNA polymerase. Total RNA from either culture cells or individual blastocysts was prepared using RNeasy Protect Mini Kit (Qiagen) and was reverse transcribed using Superscript III First-Strand Synthesis System (Invitrogen). Ex Taq Hot Start version (Takara, Shiga, Japan) was used in the PCR reactions.

2.5. Statistical analyses

Proportions were compared by a simple two-tailed z test not requiring the square root and arc sin transformation.

3. Results and discussion

3.1. Oct4 and Cdx2 expression in donor cells

Donor cells from adult mice as well as ES cells were immunostained with antibodies against Oct4 and Cdx2. Neither Oct4 nor Cdx2 expression was detected in two types of somatic cells, cumulus cells and fibroblasts (Fig. 1A and B), which is consistent with previous reports: in the adult, the expression of *Oct4* and its related genes are excluded from the somatic tissues [16,24] and *Cdx2* expression is confined to the intestinal epithelium [25]. In control experiments, as expected, ES cells expressed Oct4 but not Cdx2 (Fig. 1C) and an attached ICSI blastocyst expressed both Oct4 and Cdx2 appropriately (Fig. 1D). These immunohistochemical data were also confirmed by RT-PCR analysis (Fig. 1E). Thus, these two genes were discernibly silent in these somatic donor cells, that is, before nuclear transfer. Although *Oct4* should start to express earlier than *Cdx2* [11,13,26], both genes in the donor cells have to somehow be reactivated before blastocyst formation and after nuclear transfer for normal specification of embryonic and extraembryonic lineages.

3.2. Oct4 and Cdx2 expression in blastocysts cloned from somatic cells

At 96 h after oocyte activation following nuclear transfer, cloned blastocysts from cumulus and fibroblast cells (n = 54

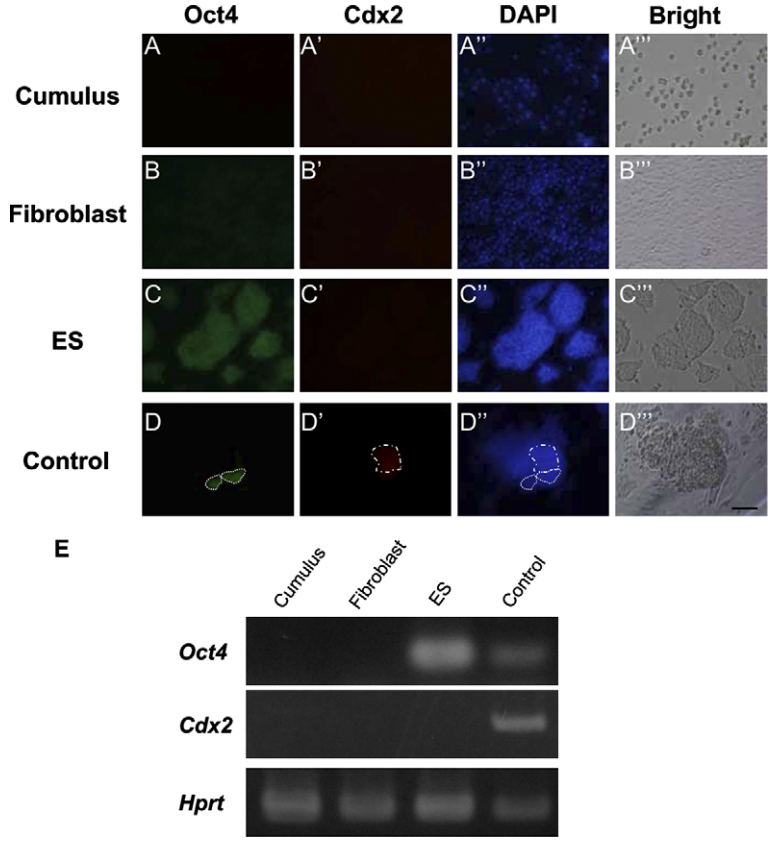

Fig. 1. Oct4 and Cdx2 expression in donor cell lines. Cumulus (A–A‴), fibroblast (B–B‴), and ES (C–C‴) cells were doubly stained with anti-Oct4 and -Cdx2 antibodies. Oct4 expressed only in ES cells. None of these cell lines expressed Cdx2. Fertilized embryos grown in vitro (D–D‴) were stained as control for these two antibodies. Oct4 and Cdx2 stained the ICM and trophoblast giant cells, respectively. Scale bar = 50 μm. (E) RT-PCR on cumulus, fibroblast, and ES cells as well as fertilized E4.5 embryos as control to detect marker gene expression. The gene expression patterns of *Oct4* and *Cdx2* analyzed by RT-PCR were consistent with the staining results by their specific antibodies.

Table 1
Classification of embryos by Oct4 and Cdx2 expression

Pattern types	Type I	Type II	Type III	Type IV	Total no.
Oct4	Normal	Low	Normal	Low	
Cdx2	Normal	Normal	Low	Low	
No. cumulus clone (%)	27 (50)[a]	23 (43)[a]	0 (0)	4 (7)[a]	54
No. fibroblast clone (%)	25 (60)[a]	17 (41)[a]	0 (0)	0 (0)[a]	42
No. ES clone (%)	50 (78)[b]	3 (5)[b]	7 (11)[a]	4 (6)[a]	64
No. ICSI blast (%)	76 (84)[b]	6 (7)[b]	5 (6)[a]	4 (4)[a]	91

Within the same column, values with different superscripts are significantly different ($P < 0.05$).

and 42, respectively) were subjected to immunocytochemistry of Oct4 and Cdx2. In this study, all the blastocysts were classified into four types, type I–type IV, according to the combinations of their expression levels (Table 1). Almost half of the cumulus and fibroblast clones (50% and 60%, respectively) showed that both Oct4 and Cdx2 expression patterns and were classified as type I (Fig. 2A and D). However, it is noted that a significant number of type I blastocysts showed aberrant Oct4 expression patterning in a non-restricted manner to the ICM as described later. Therefore, not all but only some of these type I clones are expected to develop successfully following implantation. In type II cloned blastocysts, a normal level of Cdx2 but none or low level of Oct4 was observed (Fig. 2B and E), suggesting the presence of TE lineage and a lack of ICM lineage. These type II blastocysts (43% and 40%, respectively, for cumulus and fibroblast clones) are not likely to develop further as seen in *Oct4*-deficient embryos [6]. Interestingly, there were no type III somatic cloned blastocysts which should have normal Oct4 expression and low expression of Cdx2. Finally, only a few type IV cloned blastocysts (7% and 0%) were found, in which both Oct4 and Cdx2 were hardly detected (Fig. 2C). Thus, more than 90% of the somatic clones fell into either type I or II, both of which express Cdx2 (Table 1). In contrast, the number of Oct4-expressing somatic clones as included into either type I or III was only around half, suggesting normal establishment and maintenance of TE lineage in most somatic clones but difficulty in establishment and maintenance of ICM lineages. These immunohistochemical data for cumulus clones were also confirmed by RT-PCR analysis (Fig. 2F). Therefore, most of the somatic cloned blastocysts have the ability to produce the extraembryonic lineage regardless of failure to establish or maintain the embryonic lineage at the blastocyst stage.

3.3. Blastocysts cloned from embryonic stem cells vs. somatic cells

ES cloned blastocysts were examined as well as embryos fertilized by ICSI ($n = 64$ and 91, respectively) in the same manner as the somatic clones. As expected, a greater number of type I blastocysts were found in ES clones as well as ICSI control embryos (78% and 83%, respectively, for ES clones and ICSI control). Type II (5% and 7%) and IV (6% and 4%) blastocysts were very limited. Interestingly, a minor but significant amount of type III blastocysts were found from ES clones and ICSI embryos (11% and 6%, respectively) (Fig. 3A–D). Thus, about 80% of cloned blastocysts from ES cells normally expressed both Oct4 and Cdx2 as well as fertilized embryos, in agreement with previous reports of *Oct4* expression in ES clones [15–17]. Taken together with the results for the somatic clones, the majority of cloned blastocysts belong to either type I or II, suggesting the successful reactivation of *Cdx2* in most clones is likely and yet independent of donor cell type but the efficiency of successful reactivation of *Oct4* depends on origins of the donor nuclei (Table 1). Recently it is suggested that *Oct4* expression is differently regulated, depending on the developmental stages and cell types, by the different epigenetic statuses of the *Oct4* gene including DNA-methylation and chromatin remodeling [27]. Therefore, this dependency of *Oct4* reactivation on donor cells in cloned embryos may reflect the epigenetic status of *Oct4* in each donor cell type.

3.4. Non-restricted expression of Oct4 in cloned embryos

Oct4 expression patterning in the ICM is often disturbed in cloned embryos [15,17]. We also found cloned blastocysts in which Oct4-positive cells were not restricted to the ICM region regardless of donor cell type (Fig. 4B). Next, we examined the frequency of this disturbance among type I cloned embryos. A significantly lower number of Oct4-restricted expressing blastocysts (41% and 64% for cumulus and fibroblast clones, respectively) was observed after somatic nuclear transfer (Fig. 4C). In contrast, ES clones and fertilized embryos showed a high percentage of Oct4-restricted expression patterns (87% and 86%, respectively). Thus, not only are Oct4 expression levels but also the patterning unique to individual donor cell types. Importantly, regardless of restriction of Oct4 expression, Cdx2 was normally expressed in almost all the presumptive TE cells (Fig. 4B). Actually, in more than 90% of Oct4 expressing cells in presumptive TE cells of ''non-restricted'' cloned blastocysts, Cdx2 expression was also more or less maintained (Fig. 4B, arrows), suggesting an abnormal gene regulation of *Oct4* and *Cdx2* in presumptive TE cells as well as a compromised or delayed lineage determination which would prevent normal embryonic development. However, this abnormal Oct4 co-expression with Cdx2 in presumptive TE cells is not expected to be maintained long-term because of mutual inhibition between Oct4 and Cdx2 [13,14]. Actually, when cumulus clones with such non-restricted Oct4 expression are cultured for outgrowth formation, half lack Oct4-expressing cells [15], suggesting the inactivation of *Oct4* expression by Cdx2 in subsequent culturing.

In this study, we have revealed that most cloned blastocysts regardless of donor origin, express Cdx2 normally despite loss or aberrancy of Oct4 expression, suggesting a capability of clones to reactivate *Cdx2* and to correctly specify the extraembryonic lineage, even when Oct4 expression occurs simultaneously in presumptive TE cells. Therefore, it is likely that

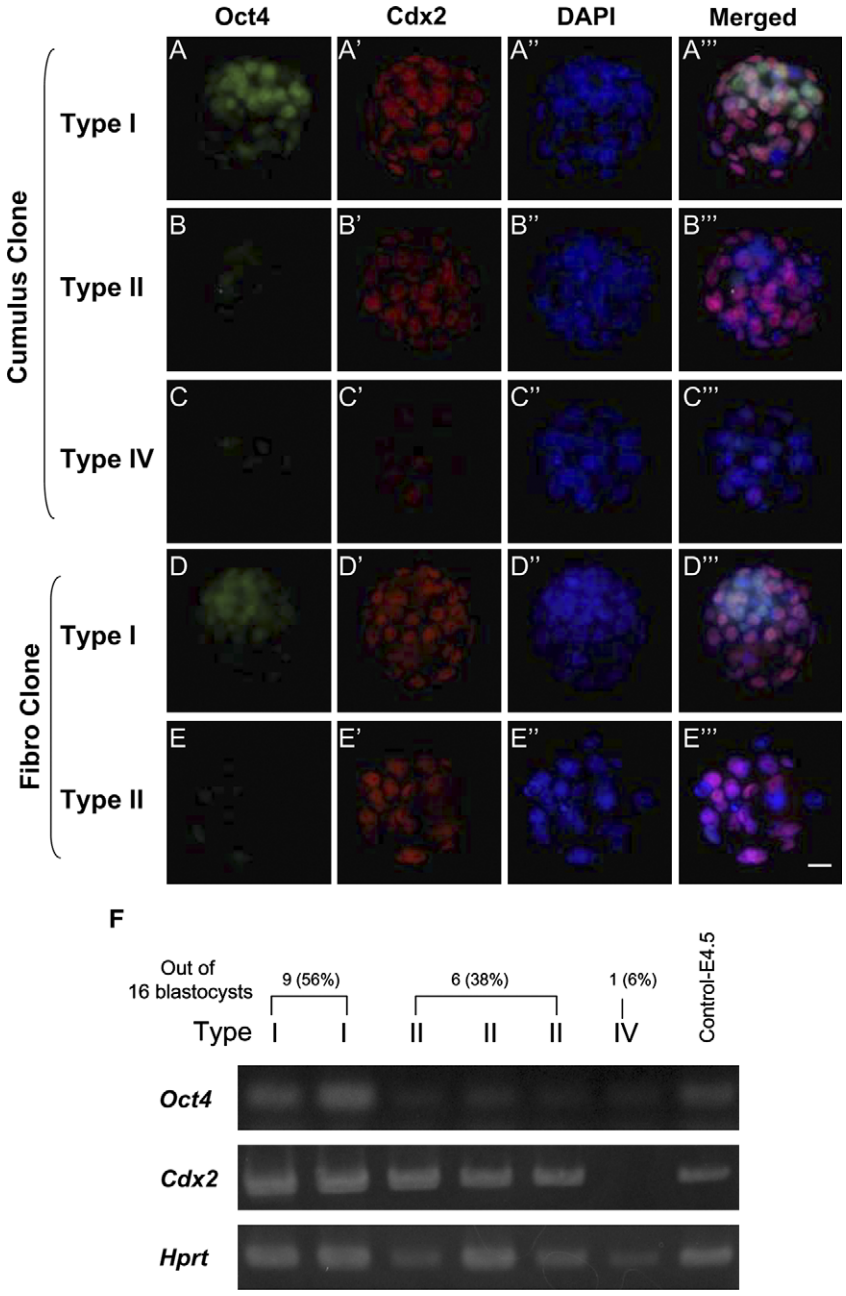

Fig. 2. Oct4 and Cdx2 expression patterns in the somatic cloned blastocysts. Blastocysts cloned from cumulus (A–C) or fibroblast (D, E) cells were doubly stained with antibodies against Oct4 (green) and Cdx2 (red). Those blastocysts were classified as four types (type I–type IV) as shown in Table 1 according to the protein level of Oct4 and Cdx2. Type I (A–A‴ and D–D‴) expressed both Oct4 and Cdx2. In type II cloned blastocysts (B–B‴ and E–E‴), Cdx2 expression was normal but Oct4 was nearly undetected. There were no type III somatic cloned blastocysts found in which only Oct4 is highly expressed while Cdx2 expression is low. Type IV blastocysts show low expression of both Oct4 and Cdx2. Scale bar = 25 μm. (F) Representative examples of RT-PCR on individual E4.5 blastocysts cloned from cumulus cells. Classification of 16 embryos based on *Oct4* and *Cdx2* expression as well as the rates for each type which are comparable to those by immunohistochemical analysis shown in Table 1.

Oct4 is preferentially erroneously reprogrammed rather than inappropriately expressed which would reflect a total failure of reprogramming itself. In other words, in somatic clones, individual genes essential for embryonic development, are not expected to be concurrently reprogrammed but rather differently, which may represent different mechanisms in reprogramming of these individual genes after SCNT like DNA-methylation and chromatin remodeling dependent [27] and independent mechanisms.

Although cloned mice routinely show abnormal non-lethal placental development [19,28], placentas alone are often found in the absence of an embryo proper at full term [20,29,30], consistent with our finding that the extraembryonic lineage is established and maintained in most somatic clones. Our find-

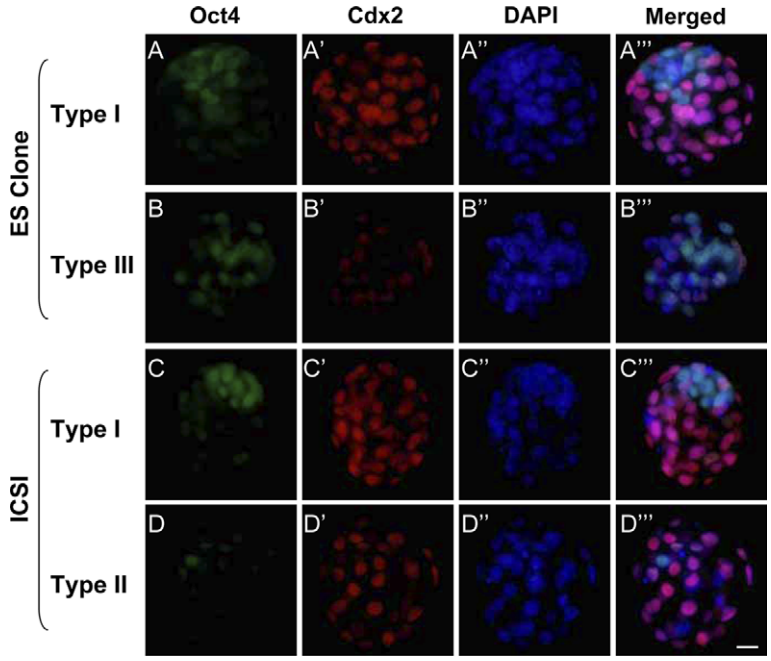

Fig. 3. Oct4 and Cdx2 expression patterns in ES clones and fertilized embryos. (A, B) Oct4 and Cdx2 expression in blastocysts cloned from ES cells. Blastocysts cloned from ES cells (A, B) and fertilized embryos (C, D) were doubly stained with antibodies against Oct4 (green) and Cdx2 (red). Among ES clones or fertilized embryos, we found type III embryos which highly express Oct4 but minimally express Cdx2. Scale bar = 25 μm.

ings also lead to an interesting possibility that the establishing rates of trophoblast stem cells [31] even from cloned blastocysts (NT-TS cells) should be comparable to those from fertilized blastocysts unlike the establishment of NT-ES cells. Thus, our findings provide insight into both the reprogramming of somatic nuclei and embryonic development of somatic clones.

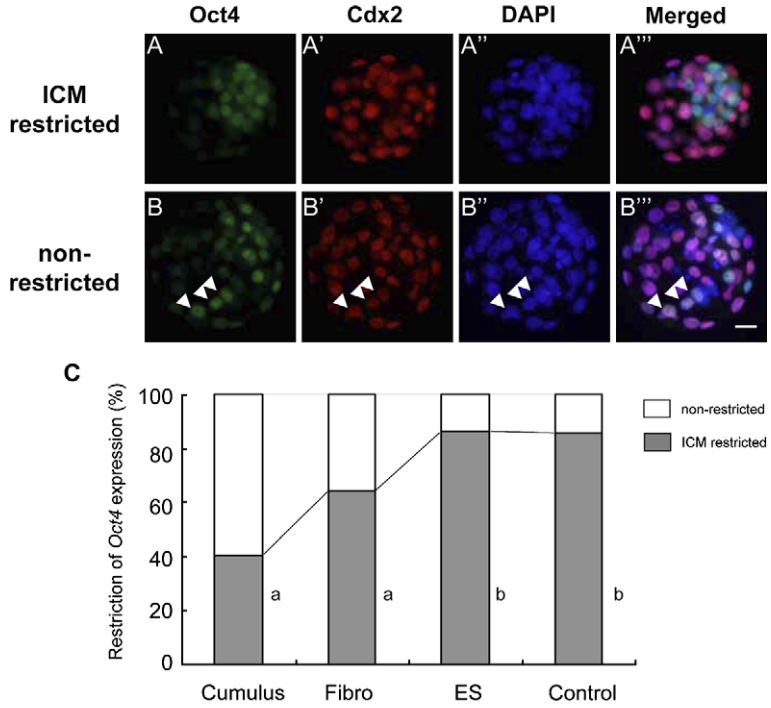

Fig. 4. Oct4 restricted and non-restricted expressions in clones. Even when Oct4 is highly expressed, the expression pattern was often abnormal in the somatic clones. Oct4-positive cells were not always restricted to the ICM but rather dispersed among the TE cells (B–B‴). Mostly, those cells were also Cdx2-positive (arrows). Scale bar = 25 μm. (C) The rates of restriction of Oct4 positive cells were dependent upon the donor cell lines.

Acknowledgments: We acknowledge discussions with Ms. T. Castranio. We also thank K. Tachibana and Y. Sakaide for their technical assistance. We are grateful to the Laboratory for Animal Resources and Genetic Engineering for housing the mice. These studies were supported by RIKEN (Strategic Program for Research and Development (FY2005) to S.K.) and MEXT (17780213 to S.K. and 15080211, 13GS0008, 15681014 and a project for the realization of regenerative medicine to T.W.).

References

[1] Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C.F., Studer, L. and Mombaerts, P. (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science 292, 740–743.

[2] Wakayama, S., Kishigami, S., van Thuan, N., Ohta, H., Hikichi, T., Mizutani, E., Yanagimachi, R. and Wakayama, T. (2005) Propagation of an infertile hermaphrodite mouse lacking germ cells by using nuclear transfer and embryonic stem cell technology. Proc. Natl. Acad. Sci. USA 102, 29–33.

[3] Wakayama, S., Ohta, H., Kishigami, S., van Thuan, N., Hikichi, T., Mizutani, E., Miyake, M. and Wakayama, T. (2005) Establishment of male and female nuclear transfer embryonic stem cell lines from different mouse strains and tissues. Biol. Reprod. 72, 932–936.

[4] Wakayama, S., Mizutani, E., Kishigami, S., Van Thuan, N., Ohta, H., Hikichi, T., Thuy, H.B., Miyake, M. and Wakayama, T. (2005) Mice cloned by nuclear transfer from somatic and ntES cells derived from the same individuals. J. Reprod. Dev. 51, 765–772.

[5] Wakayama, T. and Yanagimachi, R. (1999) Cloning the laboratory mouse. Semin. Cell Dev. Biol. 10, 253–258.

[6] Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H.R. and Smith, A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379–391.

[7] Niwa, H., Miyazaki, J. and Smith, A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. 24, 372–376.

[8] Palmieri, S.L., Peter, W., Hess, H. and Schöler, H.R. (1994) Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev. Biol. 166, 259–267.

[9] Adjaye, J., Huntriss, J., Herwig, R., BenKahla, A., Brink, T.C., Wierling, C., Hultschig, C., Groth, D., Yaspo, M.L., Picton, H.M., Gosden, R.G. and Lehrach, H. (2005) Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. Stem Cells 23, 1514–1525.

[10] Hay, D.C., Sutherland, L., Clark, J. and Burdon, T. (2004) Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells 22, 225–235.

[11] Strumpf, D., Mao, C.A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132, 2093–2102.

[12] Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F. and Beck, F. (1997) Homeosis and intestinal tumours in *Cdx2* mutant mice. Nature 386, 84–87.

[13] Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, Y. and Rossant, J. (2005) Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation. Cell 123, 917–929.

[14] Tolkunova, E., Cavaleri, F., Eckardt, S., Reinbold, R., Christenson, L.K., Schöler, H.R. and Tomilin, A. (2006) The caudal-related protein Cdx2 promotes trophoblast differentiation of mouse ES cells. Stem Cells 24, 139–144.

[15] Boiani, M., Eckardt, S., Schöler, H.R. and McLaughlin, K.J. (2002) Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev. 16, 1209–1219.

[16] Bortvin, A., Eggan, K., Skaletsky, H., Akutsu, H., Berry, D.L., Yanagimachi, R., Page, D.C. and Jaenisch, R. (2003) Incomplete reactivation of *Oct4*-related genes in mouse embryos cloned from somatic nuclei. Development 130, 1673–1680.

[17] Boiani, M., Gentile, L., Gambles, V.V., Cavaleri, F., Redi, C.A. and Schöler, H.R. (2005) Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments. Stem Cells 23, 1089–1104.

[18] Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369–374.

[19] Wakayama, T. and Yanagimachi, R. (1999) Cloning of male mice from adult tail-tip cells. Nat. Genet. 22, 127–128.

[20] Wakayama, T., Rodriguez, I., Perry, A.C.F., Yanagimachi, R. and Mombaerts, P. (1999) Mice cloned from embryonic stem cells. Proc. Natl. Acad. Sci. USA 96, 14984–14989.

[21] Kishigami, S., Wakayama, S., Van Thuan, N. and Wakayama, T. (2004) Similar time restriction for intracytoplasmic sperm injection and round spermatid injection into activated oocytes for efficient offspring production. Biol. Reprod. 70, 1863–1869.

[22] Kishigami, S., Van Thuan, N., Hikichi, T., Ohta, H., Wakayama, S., Mizutani, E. and Wakayama, T. (2006) Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids. Dev. Biol. 289, 195–205.

[23] Goldin, S.N. and Papaioannou, V.E. (2003) Paracrine action of FGF4 during periimplantation development maintains trophectoderm and primitive endoderm. Genesis 36, 40–47.

[24] Yeom, Y.I., Fuhrmann, G., Ovitt, C.E., Brehm, A., Ohbo, K., Gross, M., Hubner, K. and Schöler, H.R. (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122, 881–894.

[25] James, R., Erler, T. and Kazenwadel, J. (1994) Structure of the murine homeobox gene *cdx-2*. J. Biol. Chem. 269, 15229–15237.

[26] Wang, Q.T., Piotrowska, K., Ciemerych, M.A., Milenkovic, L., Scott, M.P., Davis, R.W. and Zernicka-Goetz, M. (2004) A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. Dev. Cell 6, 133–144.

[27] Hattori, N., Nishino, K., Ko, Y.G., Hattori, N., Ohgane, J., Tanaka, S. and Shiota, K. (2004) Epigenetic control of mouse *Oct-4* gene expression in embryonic stem cells and trophoblast stem cells. J. Biol. Chem. 279, 17063–17069.

[28] Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R. and Shiota, K. (2001) Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. Biol. Reprod. 65, 1813–1821.

[29] Ogura, A., Inoue, K., Ogonuki, N., Lee, J., Kohda, T. and Ishino, F. (2002) Phenotypic effects of somatic cell cloning in the mouse. Cloning Stem Cells 4, 397–405.

[30] Inoue, K., Wakao, H., Ogonuki, N., Miki, H., Seino, K., Nambu-Wakao, R., Noda, S., Miyoshi, H., Koseki, H., Taniguchi, M. and Ogura, A. (2005) Generation of cloned mice by direct nuclear transfer from natural killer T cells. Curr. Biol. 15, 1114–1118.

[31] Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A. and Rossant, J. (1998) Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072–2075.